PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: Q72135
Tadashi YONEDA, et al.	
Appln. No.: 10/528,773	Group Art Unit: 1651
Confirmation No.: 6335	Examiner: Thane E. Underdahl
Filed: March 23, 2005	

For: PRODUCTION METHOD FOR ITURIN A AND ITS HOMOLOGUES

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Tadashi YONEDA, hereby declare and state:

THAT I am one of the inventors of the invention disclosed and claimed in the above-identified application;

THAT I am a citizen of JAPAN;

THAT I have received the degree of Master of Engineering in Division of Fermentation technology from Osaka University Graduate School of Engineering;

THAT I have been employed by SHOWA DENKO K. K. since April 1991, where I hold a position as a researcher, with responsibility for research of enzymes (April 1991 to December 1997), research of functional chemicals (January 1998 to May 1998), research of surfactin and iturin (June 1998 to December 2006) and research of environmental biotechnology (January 2007 to the present);

THAT I am familiar with the prosecution history of the above-identified application. I carefully reviewed the Office Action dated February 27, 2009, issued in the instant application, and am familiar with the rejection of Claims 1-9 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Phae *et al.*, in view of Tulin *et al.* (*Biotechnology and Bioengineering*, 1992, 40:844-850), DeMain *et al.* (*J. Bacteriol.*, 1958, 75(5):517-522) and Gary *et al.* (*J. Bacteriol.*, 1952, 64(4):501-512), and the rejection of Claims 1-10 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Phae *et al.*, in view of Tulin *et al.* (*Biotechnology and Bioengineering*, 1992, 40:844-850), DeMain *et al.* (*J. Bacteriol.*, 1958, 75(5):517-522) and Gary *et al.* (*J. Bacteriol.*, 1952, 64(4):501-512), as applied above, and further in view of Ohno #1.

The present Declaration is submitted in support of Applicants' position that the *B. subtilis* NB22 strain of Phae *et al.* is neither the same strain as *B. subtilis* SD142, nor does it possess substantially the same properties. Rather, as demonstrated below, in contradistinction to the *B. subtilis* NB22 strain, which produces surfactin, *B. subtilis* SD142 does not.

Although surfactin and Iturin A are structurally similar lipopeptide compounds, surfactin and Iturin A cannot readily be identified using the same HPLC conditions because surfactin possesses a high concentration of hydrophobic amino acids, whereas Iturin A possesses a high concentration of hydrophilic amino acids. As a consequence, surfactin cannot be eluted under the same conditions of Iturin A, and use of a mobile-phase having a higher elution efficiency is required to detect surfactin.

In view of the differences in hydrophobicity between surfactin and Iturin A, the conditions used to detect Iturin A (namely, under HPLC conditions using an

acetonitrile:ammonium acetate(10 mM) ratio of 3 : 4) differ from the conditions used to detect surfactin (namely, under HPLC conditions using an acetonitrile:water:trifluoroacetic acid ratio of 800:200:0.2). As shown below, I was able to detect surfactin production by *B. subtilis* NB22 when cultured in a liquid medium containing soybean powder, but not with *B. subtilis* SD142. The experimental protocol and results were as follows.

Experimental Protocol

a) *B. subtilis* SD142 and *B. subtilis* NB22 were streak-cultured in an L-plate medium at 35°C overnight. The L-plate medium consisted of 1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 1.8% agar.

b) A loopful of each cultured strain (i.e., *B. subtilis* SD142 and *B. subtilis* NB22) was inoculated into 1 ml of a liquid medium containing soybean powder, and cultured at 300 rpm and 30°C for three days. The liquid medium containing soybean powder consisted of the following:

soybean powder	8 %
maltose	6.7 %
KH_2PO_4	0.5 %
$MgSO_4$/7aq	0.05 %
$FeSO_4$/5aq	25 ppm
$MnSO_4$/5aq	22 ppm
$CaCl_2$/2aq	184 ppm

e) Following this incubation, the culture solutions were centrifuged and the supernatant was diluted five-fold with a mobile-phase solution and subjected to HPLC to determine the quantity of surfactin produced by *B. subtilis* SD142 and *B. subtilis* NB22. A Surfactin reagent (Sigma-Aldrich Corporation) was used as a standard preparation, and a 1000 ppm solution mobile phase was used to determine quantity.

The HPLC conditions were as follows:

Column:	ODS column; SHODEX C18P 4E (Showa Denko K.K.)
Mobile phase:	Acetonitrile : water : trifluoroacetic acid = 800 : 200 : 0.2
Column temperature:	40°C
Flow rate:	1.0 ml/min
Detection:	UV 205 nm
Sample amount:	5 µl

Results

As shown in the Table below, under the above HPLC conditions, surfactin was clearly produced by *B. subtilis* NB22, and accumulated in the culture media to a concentration of 710 ppm. In contrast, however, under the very same conditions, surfactin production by *B. subtilis* SD142 was not detectable (0 ppm).

Strain	Surfactin Concentration
SD142	0 ppm
NB22	710 ppm

The above experiment clearly shows that, unlike *B. subtilis* SD142, which does not produce surfactin, the *B. subtilis* NB22 strain, as used in Phae *et al.*, produces surfactin, and accumulates it in the culture medium to a level exceeding 50 ppm (i.e., 710 ppm).

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: June 9, 2009

By: Tadashi Yoneda
Name: Tadashi YONEDA